Forensic Science International 262 (2016) 233–241

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

The influences of cultivation setting on inflorescence lipid distributions, concentrations, and carbon isotope ratios of *Cannabis sp.*

Brett J. Tipple [a,b,*], Bastian Hambach [a,1], Janet E. Barnette [a], Lesley A. Chesson [c], James R. Ehleringer [a,b]

[a] Department of Biology, University of Utah, Salt Lake City, UT 84112, United States
[b] Global Change and Sustainability Center, University of Utah, Salt Lake City, UT 84112, United States
[c] IsoForensics Inc., Salt Lake City, UT, 84108, United States

ARTICLE INFO

Article history:
Received 21 November 2015
Received in revised form 3 March 2016
Accepted 8 March 2016
Available online 22 March 2016

Keywords:
Marijuana
Eradicated specimens
Stable isotopes
Compound-specific isotope analysis
n-Alkanes
Growth setting

ABSTRACT

While much is known about how the growth environment influences many aspects of floral morphology and physiology, little is known about how the growth setting influences floral lipid composition. We explored variations in paraffin wax composition in *Cannabis sp.*, a cash crop grown both indoors and outdoors across the United States today. Given an increased focus on regulation of this crop, there are additional incentives to certify the setting of *Cannabis* cultivation. To understand the impacts of the growth environment, we studied distributions, concentrations, and carbon isotope ratios of *n*-alkanes isolated from *Cannabis sp.* inflorescences to assess if variations within these lipid parameters were related to known growth settings of specimens seized by federal agents. We found that *Cannabis* plants cultivated under open-field settings had increased inflorescence paraffin wax abundances and greater production of lower molecular weight *n*-alkanes relative to plants grown in enclosed environments. Further, the carbon isotope ratios of *n*-C_{29} from *Cannabis* plants grown in enclosed environments had relatively lower carbon isotope ($\delta^{13}C$) values compared to plants from open-field environments. While this set of observations on seized plant specimens cannot address the particular driver behind these observations, we posit that (a) variations in irradiance and/or photoperiod may influence the distribution and concentration of inflorescence lipids, and (b) the $\delta^{13}C$ value of source CO_2 and lipid concentration regulates the $\delta^{13}C$ values of inflorescence *n*-C_{29} and bulk *Cannabis* plant materials. Nonetheless, by using a cultivation model based on $\delta^{13}C$ values of *n*-C_{29}, the model correctly identified the growth environment 90% of time. We suggest that these lipid markers may be used to trace cultivation methods of *Cannabis sp.* now and become a more powerful marker in the future, once the mechanism(s) behind these patterns is uncovered.

1. Introduction

The policies surrounding the use and distribution of marijuana (*Cannabis sp.*) are controversial within the United States. While possession, cultivation, and sales of marijuana remain illegal under the Federal Controlled Substance Act, the District of Columbia and the States of Washington, Colorado, Oregon, and Alaska have recently legalized marijuana for personal use and additional States have current ballot measures. In response, the Department of Justice released a series of enforcement priorities seeking to avert the public health consequences of marijuana usage; curb trafficking and violence associated with illegal marijuana distribution and sales by criminal enterprises; and limit transport of marijuana between jurisdictions with differing marijuana laws. These District and State jurisdictions are now working to develop regulatory mechanisms for the production and sales of marijuana and other marijuana-derived products; however, given the nascent state of the legislation, there remain numerous ambiguities within these regulations. In particular, within jurisdictions where *Cannabis* production and sale are legal, the growth environments of *Cannabis* cultivation are highly regulated by the local government. As an example, the State of Colorado requires the physical locations of *Cannabis* cultivation—such as individual fields or specific glass/hothouses—as well as the

* Corresponding author at: Department of Biology, University of Utah, Salt Lake City, UT 84112, United States. Tel.: +1 18015813545.
E-mail addresses: brett.tipple@utah.edu (B.J. Tipple), b.hambach@noc.soton.ac.uk (B. Hambach), janet.barnette@utah.edu (J.E. Barnette), lesley@isoforensics.com (L.A. Chesson), jim.ehleringer@utah.edu (J.R. Ehleringer).
[1] Current institution: National Oceanography Centre, University of Southampton, Southampton, United Kingdom.

http://dx.doi.org/10.1016/j.forsciint.2016.03.029
0379-0738/

site of production facilities to be certified and all crops and products must be inventoried. Thus, there is a need for product traceability during plant cultivation, harvest, shipment, and following the manufacture of *Cannabis* products.

Stable isotope analysis of marijuana has demonstrated its potential to improve the forensic and law enforcement communities' understanding of marijuana production methods, growth environments, and trafficking networks [1–3]. In this respect, carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values have proven moderately useful. In a series of papers, Shibuya and colleagues demonstrated the potential to differentiate three of the five major production regions of marijuana cultivation in Brazil based on observed differences in the bulk $\delta^{13}C$ and $\delta^{15}N$ isotope values of seized marijuana samples [4,5]. West and others followed with a study of eradicated and seized material from the U.S., but could not distinguish region-of-origin based on bulk $\delta^{13}C$ and $\delta^{15}N$ values alone [6]. While cultivation location could not be assigned in this dataset, the growth environment could be identified using $\delta^{13}C$ values as plants grown outdoors had unique values compared to plants grown in a greenhouse system [6].

In plants, stable carbon isotope ($\delta^{13}C$) values reflect the additive influences of the $\delta^{13}C$ value of atmospheric CO_2 and isotopic fractionations associated with diffusion and carbon fixation [7,8]. These fractionation events depend on the ratio of the concentrations of atmospheric CO_2 inside (c_i) and outside (c_a) of the leaf. Given that the $\delta^{13}C$ value of atmospheric CO_2 and plant fractionation factors are relatively fixed, c_i/c_a is responsible for the majority of isotopic variability for a given species living in natural and managed environments [7,8]. Factors influencing a plant's $\delta^{13}C$ value through variation in c_i/c_a are broadly related to plant-water relations and irradiance [7]. The c_i/c_a is responsive to changes in the stomatal conductance, with important influences on conductance being ambient water vapor deficit, soil moisture, and leaf temperature [9–17]. Both field and laboratory studies have provided extensive evidence for the impact of plant-water relations and irradiance on $\delta^{13}C$ values in a variety of plant tissues [18–22]. However, there may be cases where bulk plant tissues are not available, particularly with drug compounds derived from plants, and there have been very few experiments carried out under semi-controlled conditions to understand how these processes effect the distributions of and $\delta^{13}C$ values of specific plant molecules [23–25].

Analysis of non-refractory *Cannabis sp.* compounds, particularly the cannabinoids and other terpenoids, has been an area of significant scientific research [26–28]. The distribution of cannabinoids has been used to discriminate between *Cannabis* strains and geographic origin of marijuana strains [29,30]. Recently, compound-specific isotope analysis (CSIA) of the carbon isotope values of cannabinol (CBN), cannabidiol (CBD) and THC has been demonstrated as feasible [31]. However, it has been well documented that the distribution of cannabinoids can vary markedly within a single plant, through a plant's life cycle, as plant material ages, and within a single seizure collection [32–35]. These variations complicate the standardized usage of cannabinoid distributions and isotope ratios of these compounds as regulatory tools and illustrate the need for the development of a method using refractory, unchanging compounds to monitor and source *Cannabis* compounds.

High molecular weight straight-chain alkanes (*n*-alkanes) are ubiquitous in higher plants including *Cannabis sp.* [36]. Furthermore, *n*-alkanes are highly refractory and are not altered by isotopic exchange at normal surface temperatures and pressures [37]. These characteristics make *n*-alkanes a possible tool for the regulation and certification of *Cannabis*-derived products.

Here, we present chain-length distributions, concentrations, and stable carbon isotope compositions of *n*-alkanes extracted from *Cannabis* inflorescences seized by the U.S. Drug Enforcement Agency (DEA) from clandestine growing operations employing either enclosed, greenhouse systems or open-field farming methods. This experimental design allows us to investigate the impacts of cultivation method on plant waxes and we hypothesized that cultivation method is recorded in *Cannabis* n-C_{29} carbon isotope ratios, similar to the information recorded by bulk *Cannabis* materials [6,38]. To test this hypothesis, we analyzed 84 *Cannabis* inflorescences of U.S. origin from known cultivation settings (i.e., enclosed system vs. open-field environments) and explored the association between growth settings and the distributions, concentrations, and $\delta^{13}C$ values of n-C_{29}.

2. Methods

2.1. Sample localities and materials collected

We analyzed inflorescences from 84 fully mature domestic marijuana samples of known origin from 53 counties within 18 states (Table 1). Samples analyzed here are a subset of materials used in studies by West et al. [6,39] and Hurley et al. [38,40]. In this study on compound specific isotope analyses, samples were selected from 9 states where possession and usage of marijuana is illegal (AR, FL, IN, KY, MO, TN, TX, WI, and WV) and from 9 additional states with various state-level statutes ranging from legal medical usage (HI, IL, and MT), medical and possession decriminalization (CA, NY, and VT), and legalization (AK, OR, WA). *Cannabis* inflorescence, leaf material, stems, and in some cases roots and seeds were collected between 2003 and 2006 through the U.S. Drug Enforcement Administration's (DEA) eradication efforts. Notes were provided reporting the growth setting (i.e., enclosed, open-field) employed at the clandestine growing operation for all specimens. In addition, information regarding number of plants seized, approximate canopy-cover, and plant height was reported for some, but not all samples. No information regarding the species or specific cultivar of *Cannabis* was provided. Materials used in this study were collected from archived material that was desiccated and stored in 4-ml glass vials at the University of Utah since initial sample intake. Of the 84 samples, 62 of them were noted by the DEA as having been grown in open-field environments and 22 as having been cultivated within enclosed environments.

2.2. Lipid extraction, identification, and quantification

Samples (50–400 mg) of inflorescences were isolated and pulverized with a mortar and pestle, filtering and regrinding residual large particles by passing ground material through a 250-μm stainless steel sieve until all material was ground and homogenized. Lipids were extracted from 100 to 300 mg of powdered inflorescences with 2:1 dichloromethane (DCM)/methanol by ultra-sonication (30 min × 2). The resulting total lipid extracts were concentrated under a stream of purified nitrogen using a FlexiVap Work Station (Glas-Col, Terre Haute, IN, USA), transferred to 4-ml glass vials, and further evaporated under a gentle stream of N_2 gas. Extracts were then separated into compound classes by column chromatography using 1 g deactivated silica gel (70–230 mesh) in an ashed Pasteur pipette, and eluted with 2 ml hexane to obtain the saturated hydrocarbons following Tipple and Pagani [41].

Compounds were identified and their abundances were quantified using a Thermo Ultra gas chromatograph (GC) fitted with a programmable-temperature vaporization (PTV) injector and flame ionization detector. Hydrocarbons were introduced to the PTV injector at 40 °C, followed by a 50 °C/s ramp to 320 °C. The GC oven temperature program utilized was 60–320 °C at 15 °C/min

Table 1
Concentrations, distributions, and carbon isotope values of *n*-alkanes from *Cannabis* inflorescence.

ID	Cultivation setting	State	*n*-C_{23}	*n*-C_{24}	*n*-C_{25}	*n*-C_{26}	*n*-C_{27}	*n*-C_{28}	*n*-C_{29}	*n*-C_{30}	*n*-C_{31}	*n*-C_{32}	*n*-C_{33}	ACL	CPI	*n*-C_{29}	SD	Bulk[a]	ε_{app}
			(μg/g)	(μg/g)	(μg/g)	(μg/g)	(μg/g)	(μg/g)	(μg/g)	(μg/g)	(μg/g)	(μg/g)	(μg/g)			$\delta^{13}C$ (‰, VPDB)		$\delta^{13}C$ (‰, VPDB)	(‰, VPDB)
784	Open-field	AK	1	1	12	5	138	31	565	23	100	0	0	28.8	13.7	−35.3	0.1	−27.5	−8.0
786	Enclosed	AK	1	1	35	11	374	68	1183	56	299	7	8	28.9	13.2	−37.4	0.0	−29.7	−8.0
787	Enclosed	AK	1	1	11	5	92	29	506	29	124	1	3	29.0	11.3	−56.9	1.2	−50.9	−6.4
430	Enclosed	CA	1	1	16	6	128	34	670	29	123	2	6	28.9	13.2	−28.9	0.3	−21.4	−7.6
708	Enclosed	CA	2	1	15	7	149	30	507	26	95	1	0	28.8	11.8	−36.5	0.5	−29.1	−7.6
761	Open-field	CA	1	1	23	7	254	62	1122	54	203	4	3	28.9	12.4	−35.3	0.1	−28.2	−7.3
762	Open-field	CA	0	1	6	3	75	16	449	14	64	0	0	28.9	17.6	−34.4	0.2	−26.2	−8.4
763	Open-field	CA	0	0	7	2	169	39	1155	42	185	4	4	29.0	17.5	–	–	–	–
780	Open-field	CA	1	1	23	9	239	67	1350	64	241	1	1	28.9	13.0	−35.1	0.2	−26.7	−8.6
781	Open-field	CA	2	1	20	9	271	82	1249	56	164	0	0	28.8	11.5	−33.9	0.1	−26.4	−7.7
658	Enclosed	FL	3	2	10	4	75	35	1054	49	241	4	1	29.2	14.7	–	–	–	–
308	Open-field	HI	2	1	20	11	152	60	1310	69	194	4	6	29.0	11.6	−36.6	0.1	−29.4	−7.3
502	Open-field	HI	2	2	54	18	385	108	1958	87	298	6	5	28.9	12.2	−35.6	1.2	−28.1	−7.8
663	Open-field	HI	2	1	40	18	392	73	1516	54	211	4	8	28.8	14.4	−36.8	0.0	−29.0	−8.0
664	Enclosed	HI	1	1	17	7	149	45	990	47	211	1	0	29.0	13.7	–	–	–	–
667	Open-field	HI	1	1	7	3	115	49	1084	64	220	5	4	29.1	11.8	−36.2	0.2	−28.1	−8.3
668	Open-field	HI	0	0	8	4	51	13	198	13	63	3	2	29.0	9.5	−37.1	0.1	−28.9	−8.4
669	Open-field	HI	1	0	9	2	73	15	244	12	60	1	2	28.9	12.4	−36.0	0.0	−28.2	−8.0
670	Open-field	HI	3	2	113	20	388	34	421	18	64	2	2	27.9	13.1	−38.3	0.2	−29.7	−8.9
676	Open-field	HI	1	1	15	7	159	43	633	28	103	3	2	28.8	11.1	−35.7	0.3	−28.6	−7.4
710	Open-field	HI	2	0	27	7	258	61	1456	59	216	0	0	28.9	15.4	−35.7	0.3	−27.1	−8.8
712	Enclosed	HI	1	1	7	5	52	18	372	21	79	0	0	29.0	10.9	−33.8	0.3	−26.9	−7.1
753	Open-field	HI	1	1	32	8	261	39	821	37	246	4	2	28.9	15.3	−34.9	0.0	−27.1	−8.0
754	Open-field	HI	1	1	32	8	239	36	682	30	204	5	7	28.8	14.5	–	–	–	–
428	Open-field	IL	1	1	18	6	154	45	1155	34	155	0	0	28.9	17.1	−35.4	0.0	−25.8	−9.9
264	Open-field	IN	0	1	8	4	87	19	421	14	76	6	0	28.9	13.6	−38.4	0.3	−29.8	−9.0
288	Enclosed	IN	1	1	8	3	85	26	437	19	87	1	2	29.0	12.3	−38.1	0.2	−30.1	−8.2
302	Enclosed	IN	1	1	9	5	132	55	1184	57	246	4	7	29.1	12.9	−36.2	0.1	−30.9	−5.4
343	Enclosed	IN	1	1	8	3	87	26	552	29	129	2	3	29.1	12.8	−54.1	0.1	−45.9	−8.6
349	Open-field	IN	0	1	7	3	66	17	436	14	78	1	0	29.0	17.3	−34.9	0.0	−25.8	−9.4
363	Open-field	IN	0	1	20	8	205	26	484	14	66	1	2	28.5	15.4	−37.5	1.8	−28.2	−9.6
379	Open-field	IN	1	1	20	8	178	34	581	25	98	0	0	28.7	12.9	−34.0	0.2	−26.1	−8.1
483	Open-field	IN	2	1	63	20	698	91	1590	58	329	7	8	28.6	15.1	−36.9	0.9	−28.0	−9.1
501	Open-field	IN	2	1	31	7	127	21	265	11	32	0	0	28.3	11.4	−38.8	0.2	−29.4	−9.7
513	Enclosed	IN	1	1	24	10	205	49	869	37	146	3	4	28.8	12.5	−36.6	0.1	−29.2	−7.6
514	Open-field	IN	26	3	36	10	316	97	2427	139	487	10	10	29.0	12.7	−36.0	0.0	−28.1	−8.2
532	Open-field	IN	2	2	49	14	336	41	649	24	127	2	3	28.5	14.1	−35.7	0.0	−27.6	−8.3
534	Open-field	IN	1	1	27	7	242	36	686	29	195	4	4	28.8	15.0	−38.3	0.4	−28.8	−9.7
536	Open-field	IN	2	1	47	13	321	49	796	28	114	2	3	28.5	13.9	−37.2	0.2	−28.9	−8.6
542	Open-field	IN	2	1	32	9	336	76	1558	67	321	5	9	28.9	14.3	−36.4	0.1	−28.1	−8.5
548	Open-field	IN	3	2	50	11	198	30	523	15	63	1	1	28.4	13.9	−35.9	0.2	−27.4	−8.8
682	Open-field	KY	2	1	49	16	504	82	1324	73	328	9	10	28.8	12.2	−35.3	0.3	−27.2	−8.4
683	Open-field	KY	1	1	8	3	78	25	405	17	62	0	0	28.9	12.0	−37.5	0.0	−28.5	−9.3
700	Open-field	KY	2	2	40	13	394	69	1068	49	269	0	0	28.8	13.3	−36.5	0.3	−27.4	−9.3
434	Open-field	MO	1	0	3	1	33	22	558	62	262	13	9	29.6	8.7	−40.1	0.2	−29.2	−11.3
556	Open-field	MO	1	1	16	6	205	35	697	24	140	2	3	28.8	15.6	–	–	–	–
557	Open-field	MO	2	1	34	10	312	44	770	27	117	6	0	28.6	14.0	−36.0	0.1	−27.6	−8.6
558	Open-field	MO	1	1	15	5	173	46	758	41	143	4	2	28.9	11.4	–	–	–	–
559	Open-field	MO	13	2	41	12	415	76	1479	63	368	8	12	28.9	14.3	−34.7	0.6	−27.9	−7.1
562	Open-field	MO	2	1	77	28	605	107	1606	71	362	6	10	28.7	12.4	−34.4	0.1	−27.2	−7.4
565	Open-field	MO	1	1	22	7	180	27	596	18	94	2	3	28.7	16.5	−36.1		−26.9	−9.4
592	Open-field	MO	36	5	264	36	1296	111	2058	71	410	9	9	28.3	17.4	−38.2	0.1	−30.3	−8.2
279	Enclosed	MT	1	1	8	4	60	22	569	32	152	3	3	29.2	12.9	−50.7	0.1	−42.5	−8.6
792	Enclosed	NY	1	1	19	6	169	37	554	22	103	3	3	28.8	12.4	–	–	–	–
268	Enclosed	OR	1	1	13	4	84	19	320	13	60	1	2	28.8	12.7	−50.9	0.2	−43.3	−7.9
269	Enclosed	OR	0	0	3	1	28	8	176	9	47	1	1	29.1	13.2	–	–	–	–
427	Open-field	OR	2	1	40	14	393	53	1104	29	139	2	5	28.6	17.1	−36.1	0.0	−27.0	−9.3
493	Open-field	OR	4	2	109	22	783	93	1957	54	256	2	8	28.5	18.0	−35.7	0.0	−27.4	−8.5
494	Open-field	OR	2	3	57	10	510	78	1645	79	221	0	0	28.7	14.3	−32.9	0.4	−28.0	−5.0
496	Open-field	OR	9	2	144	24	1013	201	4859	198	847	16	21	28.9	15.6	–	–	–	–
498	Open-field	OR	2	2	63	16	465	73	1248	44	254	5	6	28.7	14.5	−34.9	1.3	−28.4	−6.7
525	Open-field	OR	1	1	38	11	230	42	641	21	68	1	1	28.5	12.9	−37.4	0.1	−28.8	−8.8
570	Open-field	OR	1	1	40	15	230	36	508	17	48	1	1	28.4	11.9	−37.9	0.3	−28.4	−9.7
571	Open-field	OR	1	1	20	7	166	34	535	20	91	5	3	28.7	12.2	−36.5	0.3	−28.5	−8.2
598	Open-field	OR	3	2	103	23	577	78	1425	46	275	4	9	28.6	15.7	−34.8	0.0	−26.9	−8.2
599	Open-field	OR	2	1	52	12	427	56	1061	36	164	2	4	28.6	15.9	−35.3	0.2	−26.2	−9.3
600	Open-field	OR	5	3	211	41	1295	148	2887	110	716	15	20	28.6	16.2	−38.8	0.3	−30.4	−8.7
601	Open-field	OR	1	1	22	9	215	54	884	40	167	4	5	28.9	11.9	−37.5	0.0	−29.3	−8.4
401	Open-field	TN	1	0	8	2	74	16	437	16	89	3	4	29.0	16.0	−36.3	0.1	−28.5	−7.9
417	Open-field	TN	1	1	5	2	47	13	275	13	45	0	0	28.9	13.0	−36.7	0.1	−29.0	−7.9
458	Open-field	TN	2	1	17	7	184	49	1043	36	179	3	3	28.9	14.6	−33.7	1.3	−29.0	−4.8
477	Open-field	TN	1	1	12	5	96	32	980	45	172	4	5	29.1	14.6	−37.9	0.1	−28.2	−10.0
509	Open-field	TN	2	2	10	7	80	26	580	23	129	2	4	29.1	13.4	−38.9	0.1	−30.2	−9.0

Table 1 (*Continued*)

ID	Cultivation setting	State	*n*-C_{23} (μg/g)	*n*-C_{24} (μg/g)	*n*-C_{25} (μg/g)	*n*-C_{26} (μg/g)	*n*-C_{27} (μg/g)	*n*-C_{28} (μg/g)	*n*-C_{29} (μg/g)	*n*-C_{30} (μg/g)	*n*-C_{31} (μg/g)	*n*-C_{32} (μg/g)	*n*-C_{33} (μg/g)	ACL	CPI	*n*-C_{29} $\delta^{13}C$ (‰, VPDB)	SD	Bulk[a] $\delta^{13}C$ (‰, VPDB)	ε_{app} (‰, VPDB)
679	Enclosed	TX	1	0	7	4	78	23	353	22	88	2	2	29.0	10.1	–	–	–	–
365	Open-field	VT	1	1	14	3	60	14	320	10	40	0	0	28.8	15.6	−37.1	0.0	−28.4	−8.9
704	Enclosed	WA	1	1	12	6	140	41	578	31	111	3	3	28.9	10.4	–	–	–	–
705	Enclosed	WA	1	1	15	6	187	45	829	34	157	1	4	28.9	13.6	−39.9	0.5	−32.6	−7.5
706	Enclosed	WA	2	2	12	6	76	23	460	21	107	0	0	29.0	12.3	−36.3	1.2	−28.8	−7.7
429	Enclosed	WI	0	0	8	3	67	15	292	15	88	3	3	29.0	12.5	−38.7	0.2	−32.3	−6.6
499	Open-field	WI	1	1	29	13	315	53	835	28	123	2	5	28.6	13.5	−35.1	0.1	−27.6	−7.7
608	Open-field	WI	3	1	15	6	219	58	1108	46	209	4	5	28.9	13.6	−34.9	0.1	−25.8	−9.3
313	Enclosed	WV	1	1	8	4	104	20	373	17	81	2	4	28.9	12.8	−44.6	0.1	−36.9	−7.9
653	Enclosed	WV	1	1	11	4	119	27	696	30	209	5	7	29.2	15.4	–	–	–	–
656	Open-field	WV	9	3	32	9	282	54	1112	42	173	3	4	28.8	14.6	−36.0	0.4	−26.5	−9.8

[a] As reported in West et al. [6].

with a final isothermal stage lasting 30 min. Compounds were identified through comparison of elution times with *n*-alkane standards (*n*-C_{18}, *n*-C_{20}, *n*-C_{22}, *n*-C_{24}, *n*-C_{28}, and *n*-C_{32}). Compound concentrations were quantified using a 5-point calibration curve generated from reference materials (*n*-C_{24} and *n*-C_{28} analyzed together at 25 ng, 50 ng, 100 ng, 500 ng, 1000 ng).

2.3. Chain-length distributions and concentrations of n-alkanes

Peak areas of high molecular weight *n*-alkanes were measured in order to quantify distributions of *n*-alkanes. Carbon preference indices (CPI) were calculated following Marzi et al. [42]:

$$\mathrm{CPI} = \frac{(A_{23}+A_{25}+A_{27}+A_{29}+A_{31}+A_{33}) + (A_{25}+A_{27}+A_{29}+A_{31}+A_{33}+A_{35})}{2(A_{24}+A_{26}+A_{28}+A_{30}+A_{32}+A_{34})} \quad (1)$$

where "*A*" represents the area of the individual *n*-alkane peak from the chromatograph trace.

To calculate the average chain length of *n*-alkanes from inflorescences, the following was used:

$$\mathrm{ACL} = \frac{(A_{23}(23)) + (A_{25}(25)) + (A_{31}(27)) + (A_{29}(29)) + (A_{31}(31)) + (A_{33}(33)) + (A_{35}(35))}{(A_{23}+A_{25}+A_{27}+A_{29}+A_{31}+A_{33}+A_{35})} \quad (2)$$

2.4. Compound-specific isotope analysis

Compounds were separated using a Hewlett Packard 6890A GC employing a split-splitless injector held at a constant 310 °C with an GC oven temperature at 80 °C, followed by a 6 °C/min ramp to 320 °C with an isothermal for 12 min. A fused silica, DB-1, phase column (30 m × 0.25 mm I.D., 0.25 μm film thickness; J&W Scientific, Agilent Technologies, Santa Clara, CA, USA) was used with helium as the carrier at a flow of 1.2 ml/min. Compounds were subsequently combusted over nickel oxide, copper oxide, and platinum at 1000 °C and analyzed for carbon isotope ratios using a Thermo Finnigan Delta^{plus}XL isotope ratio mass spectrometer. Individual *n*-C_{29} isotope ratios were normalized to the VPDB scale using a two-point linear calibration of *n*-alkane standard reference materials, which had previously been standardized to the VPDB scale [*n*-C_{18} (−33.3‰) and *n*-C_{28} (−29.0‰)] and analyzed after every fifth unknown in an analytical sequence. Arndt Schimmelmann's "*Mix A4*" [*n*-C_{16} (−30.7‰), *n*-C_{17} (−31.2‰), *n*-C_{18} (−31.1‰), *n*-C_{19} (−33.2‰), *n*-C_{20} (−32.4‰), *n*-C_{21} (−29.1‰), *n*-C_{22} (−32.9‰), *n*-C_{23} (−31.8‰), *n*-C_{24} (−33.3‰), *n*-C_{25} (−28.5‰), *n*-C_{26} (−33.0‰), *n*-C_{27} (−29.6‰), *n*-C_{28} (−32.2‰), *n*-C_{29} (−30.1‰), and *n*-C_{30} (−29.9‰)] was analyzed twice in each analytical sequence ($n = 32$) and had an measured accuracy of 0.1 ‰. Precision for *n*-alkanes carbon isotope determinations was ±0.3‰ (1σ, $n = 157$), as determined from a co-injected QC reference material [5α-androstane (−30.1‰)].

All isotopic compositions are calculated following:

$$\delta = \left(\frac{R_{samp}}{R_{std}}\right) - 1 \quad (3)$$

where R represents the $^{13}C/^{12}C$ abundance ratio, and R_{samp} and R_{std} represent the sample and standard, respectively. Delta values are reported in per mil (‰) notation and are expressed relative to Vienna Pee Dee Belemnite (VPDB).

The apparent carbon isotope fractionation between *n*-C_{29} and bulk inflorescence (ε_{app}) is defined as:

$$\epsilon_{app} = \frac{(\delta^{13}C_{n\text{-}C29} + 1)}{(\delta^{13}C_{Bulk} + 1)} - 1, \quad (4)$$

The carbon isotope value of bulk inflorescence ($\delta^{13}C_{Bulk}$) used to determine ε_{app} were previously analyzed and reported in West et al. [6]. Briefly, West and colleague analyzed $\delta^{13}C_{Bulk}$ using an elemental analyzer coupled to a Thermo Scientific Delta^{plus} isotope ratio mass spectrometer and used an offset correction from an known reference material to normalize the unknown to the VPDB scale. They reported an overall precision of ±0.09‰ for $\delta^{13}C_{Bulk}$.

2.5. Statistical analysis

Statistical analysis was completed using JMP® 11 Pro (SAS; Cary, NC) and PRISM® 5.0c (Graphpad Software, Inc.; La Jolla, CA) for Mac OS X. Normality of distributions was tested with the Shapiro–Wilkes test. If the distributions were normal, then the Welch *t*-test was used to compare means at $\alpha = 0.05$. If the distributions were not normally distributed, then the Wilcoxon/Kruskal–Wallis (Rank Sums) test was used to assess differences between growth settings at $\alpha = 0.05$.

The measured $\delta^{13}C$ values of paired *n*-alkane and bulk inflorescences were compared using total least squares regressions. Regression lines were fitted to data only when the slope of the line was significantly different from 0 at the $\alpha = 0.01$ level.

3. Results and discussion

3.1. Compound distributions of n-alkanes on Cannabis inflorescence

Cannabis sp. produced *n*-C_{17} to *n*-C_{33} with *n*-C_{29} (929 ± 680 μg/g) being the most abundant homologue, approximately four-times more abundant than the next most abundant homologue, *n*-C_{27}

(254 ± 242 μg/g) and five-times more abundant than n-C_{31} (178 ± 134 μg/g) (Table 1). We noted large variation in concentrations of n-alkanes between individual specimens (Table 1). These variations are most likely due to differences in *Cannabis* species or cultivars. Nonetheless, significant differences between the concentrations of n-C_{29} for the two growth settings—enclosed versus open-field environments—were detected [Wilcoxon/Kruskal–Wallis, $W(91.2) = 3.781$, $z = -2.925$, $p = 0.0034$], where the plants grown in field environments had a significantly greater concentration of n-C_{29} (1041 ± 743 μg/g) compared to the plants grown in enclosed environments (614 ± 290 μg/g). Significant differences between the concentrations of total n-alkanes for the two growth settings were also detected [Wilcoxon/Kruskal–Wallis, $W(82.0) = 3.997$, $z = -3.098$, $p = 0.0019$]. Here, we found plants grown in field environments had a significantly greater absolute concentrations of n-alkanes (1695 ± 1214 μg/g) compared to the plants grown in enclosed environments, under potentially more controlled conditions (976 ± 432 μg/g) (Table 2).

Most research on chain-length distributions and concentrations of n-alkanes has been undertaken on hydrocarbons extracted from leaf material as leaf waxes are thought to be a major contributor to the organic fraction in geologic sediments. A recent meta-analysis of n-alkane concentrations of leaf material from 282 angiosperm species found on average angiosperms had an absolute n-alkane abundance of 506 ± 497 μg/g [43]. Here, we found inflorescences of a single angiosperm, *Cannabis sp.*, had nearly triple the absolute amount of n-alkanes compared to angiosperm leaf material. Several studies have noted that inflorescences have greater absolute abundances of n-alkanes compared to leaves on the same plant [44–46]. The reasons behind these differences in concentration of n-alkanes between floral structures and leaves are unresolved. Inflorescence vigor is critical to reproductive success, and thus the increased lipid concentrations may be a response to provide increased protection of these organs. Waxes on leaf cuticle are considered a strategy to guard against water loss and pathogens [36,47,48]. Lipids on the inflorescences may also prevent desiccation of floral components, in addition to acting as a safeguard against microbial or fungal attack or possibly to stabilize other defensive compounds on the inflorescence.

In addition, we found *Cannabis* plants grown under open-field conditions had nearly double the absolute concentration of n-alkanes on the inflorescences compared to plants grown in enclosed environments (Table 2). Previous research has shown that the concentrations of n-alkanes and other leaf lipids are affected by the plant's environment [51,53,65]. In particular, plants grown in field environments have been shown to produce a greater absolute abundance of n-alkanes than their greenhouse-grown counterparts [59]. Increased irradiance and UV-B light has been shown to increase the abundance of n-alkanes in some species [66], decrease the amount of n-alkanes in other species [67], or cause no change in n-alkane absolute amounts [68]. Some of the largest differences in the absolute amount of n-alkanes have been shown in plants grown under water-stressed or polluted condition ([65,69,70]). While the process behind the difference in n-alkane absolute abundance between plants grown in enclosed versus open-field environments cannot be specifically known in this study, a possible mechanism behind these distribution and concentration patterns may derive from changes in the activity or specificity of particular enzymes involved in fatty acid synthesis, chain elongation, and decarboxylation processes [51]. Although the specific mechanism cannot be isolated in our current study, our data from *Cannabis* inflorescences grown in different setting are consistent with these models and suggest a common production mechanism between leaf and inflorescence waxes.

We compiled peak areas of all n-alkanes present in the inflorescence of *Cannabis sp.* to quantify variations in the chain-length distribution of n-alkanes using carbon preference indices (CPI) (Table 1). Strong odd-over-even predominance of n-alkanes is a hallmark of higher plant wax distributions [36,49,50] and the distribution of n-alkanes in *Cannabis sp.* inflorescences is consistent with these previous observations of leaf waxes (Table 1). We found *Cannabis sp.* had an average CPI of 13.6 ± 1.9. In addition, we found significant differences between the growth settings [Welch t-test, $t(59.9) = 3.662$, $p = 0.0005$], where the plants grown in field environments had a larger CPI (14.0 ± 2.0) compared to the plants grown in enclosed environments (12.6 ± 1.3) (Table 2). The average chain length (ACL) of *Cannabis sp.* n-alkanes for all growth settings was 28.8 ± 0.2. However, significant differences between the growth settings was observed [Wilcoxon/Kruskal–Wallis, $W(69.9) = -4.931$, $z = 4.062$, $p < 0.0001$], where the plants grown in open-field environments had a smaller ACL (28.8 ± 0.2) compared to the plants grown in enclosed environments (29.0 ± 0.1) (Table 2).

Here, we observed *Cannabis* plants grown under field conditions had, on average, greater concentrations of n-alkanes with shorter chain lengths and a more pronounced odd-over-even character as compared to plants grown within enclosed systems (Table 2). These differences in n-alkane distributions and concentrations are possibly related to one or several specific growing conditions not quantified in this study. When contrasting *Cannabis sp.* cultivated in open-field versus enclosed environments, we would expect field grown plants to be exposed to a lower average growth temperature (i.e., subject to daily temperature fluctuations) while also exposed to increased water stress and increased irradiance. While limited research has been conducted on inflorescence waxes, previous studies on leaf waxes may provide some explanations for these wax distributions and abundances.

The chain-length distribution of n-alkanes on leaves has been linked to various environmental parameters, due to the functionality of waxes in controlling water loss [51]. Recent studies of woody tree species have shown relationships between leaf wax chain-length distributions and growth temperature, in which higher growth temperatures were correlated with increased abundances of longer n-alkane chain lengths (e.g., ACL) [41,52,53]. ACL values in forbs, grasses, shrubs, and trees have also been shown to vary with aridity, suggesting that plant water relations may additionally influence chain-length distributions [46,54–59]. In addition to elevated growth temperature and lower water stress, plants cultivated in enclosed environments likely were grown using managed light systems with lower irradiance. It is well established that fatty acid synthesis is strongly connected to illumination levels, as the pathway requires both ATP and NADPH from the light reactions [71,72]. Further, [59,73], showed that irradiance levels affected

Table 2
Average concentrations, carbon preference indices, average chain lengths, and carbon isotope values of *Cannabis* inflorescence n-alkanes between the two growth environments.

Cultivation setting	Total n-Alkane concentration (μg/g)	CPI	ACL	$\delta^{13}C_{n\text{-}C29}$ (‰, VPDB)	ε_{app} (‰, VPDB)
Enclosed	976 ± 432 (*22*)	12.6 ± 1.3 (*22*)	29.0 ± 0.1 (*22*)	−41.3 ± 8.2 (*15*)	−7.5 ± 0.9 (*15*)
Open-field	1695 ± 1214 (*62*)	14.0 ± 2.0 (*62*)	28.8 ± 0.2 (*62*)	−36.2 ± 1.5 (*57*)	−8.5 ± 1.1 (*57*)

lipid production, with plants grown under high-light natural environments producing leaf waxes with shorter chain lengths. While limited research has been carried out on inflorescence lipids, our data are consistent with these factors, as it would be expected that *Cannabis* plants cultivated in open-field environments would be exposed to a decreased average growth temperature, increased water stress, and increased irradiance. These hypothesized drivers of variations in inflorescence lipid concentrations and distribution could be more fully evaluated through multi-factor growth chamber experiments, which are beyond the scope of this study.

3.2. Carbon isotopes of n-alkanes from Cannabis inflorescences

As n-C_{29} was the most abundant n-alkane homologue extracted from *Cannabis sp.* inflorescences, we report $\delta^{13}C$ values of n-C_{29} ($\delta^{13}C_{n\text{-}C29}$) exclusively. We found the $\delta^{13}C_{n\text{-}C29}$ values of *Cannabis sp.* inflorescences ranged between −56.9‰ and −28.9‰, with an average $\delta^{13}C_{n\text{-}C29}$ value of -37.3 ± 4.4‰ ($n = 72$, Table 1). A significant difference in $\delta^{13}C$ values occurred between plants grown in enclosed environments versus open-field conditions [Welch t-test, $t(14.2) = 2.387$, $p = 0.031$]. Here the plants cultivated in open-field environments had more positive $\delta^{13}C_{n\text{-alkane}}$ values (-36.2 ± 1.5‰) compared to the plants cultivated in enclosed environments (-41.3 ± 8.2‰) (Table 2).

The large variability in $\delta^{13}C_{n\text{-}C29}$ values from plants grown in enclosed environments is most likely due to variations in the $\delta^{13}C$ values of the source CO_2 available to plants during growth. CO_2 within enclosed settings tends be more depleted in ^{13}C compared to well mixed, outdoor settings for two reasons. First, the lack of sufficient air circulation results in the build up of plant-respired CO_2, which is ^{13}C depleted relative to the ambient air. Second, to elevate plant growth, horticulturalists and agronomists tend to raise CO_2 levels within indoor growth environments through the addition of CO_2. Commercial supplemental CO_2 is most often fossil fuel- or biogenic-derived. Bottled CO_2 from either fossil fuels or biogenic processes typically has much lower $\delta^{13}C$ values compared to global atmospheric CO_2, resulting in indoor grown plants with extremely low $\delta^{13}C$ values [7]. The lowest $\delta^{13}C_{n\text{-}C29}$ values observed in this dataset were between −60‰ and −50‰ (Fig. 1) and would correspond to a source CO_2 with a $\delta^{13}C$ value of approximately −35‰ to −25‰ [60], equivalent to fossil fuel-derived CO_2 with a $\delta^{13}C$ value of −37‰ to −28‰ [61,62]. However, *Cannabis* plants grown in enclosed environments within a well-ventilated atmosphere that allows mixing of CO_2 with the external atmosphere can produce $\delta^{13}C$ values similar to plants grown in open settings. This may explain why the majority of indoor-grown plants have $\delta^{13}C_{n\text{-}C29}$ values similar to the $\delta^{13}C_{n\text{-}C29}$ values of plants grown under open-field conditions (Fig. 1).

We note that a single *Cannabis sp.* inflorescence sample from a plant assigned as having been cultivated within an enclosed environment had a $\delta^{13}C_{n\text{-}C29}$ value of −28.9‰, more positive relative to the $\delta^{13}C_{n\text{-}C29}$ values of plants grown in open-field environments (Fig. 1). There are two potential explanations for this very positive $\delta^{13}C_{n\text{-}C29}$ value. First, this plant was possibly grown in a field setting but incorrectly assigned as indoor grown in the DEA records during confiscation and eradication. A second possibility is this plant was grown in bottled CO_2 that had been derived from volcanic or geothermal sources [63].

3.3. Apparent fractionation between inflorescence n-alkanes and bulk Cannabis inflorescences

The $\delta^{13}C$ values of bulk inflorescence ($\delta^{13}C_{Bulk}$) linearly correlated with the $\delta^{13}C_{n\text{-}C29}$ values collected from the same samples (Fig. 2). A total least squares regression line fitted to the paired data was described by the equation:

$$\delta^{13}C_{n-C29} = 0.95 \times \delta^{13}C_{Bulk} - 9.6‰ (r^2 = 0.94,\ F_{1,71} = 1149.2,\ p < 0.0001), \quad (5)$$

The slope was not different than 1 at $\alpha = 0.01$. Total least squares regression lines fitted to the paired data separated by enclosed versus open-field settings were described by the equations:

$$\text{Enclosed}: \delta^{13}C_{n-C29} = 1.0 \times \delta^{13}C_{Bulk} - 7.2‰ (r^2 = 0.99,\ F_{1,14} = 1304.4,\ p < 0.0001), \quad (6)$$

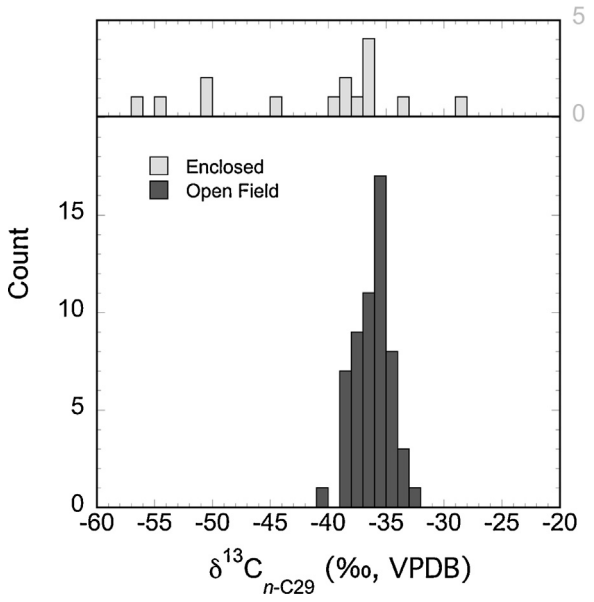

Fig. 1. (A) Histograms of carbon isotope ratios of n-C_{29} extracted from domestic *Cannabis sp.* inflorescences grown within enclosed environments (light gray) and field conditions (dark gray).

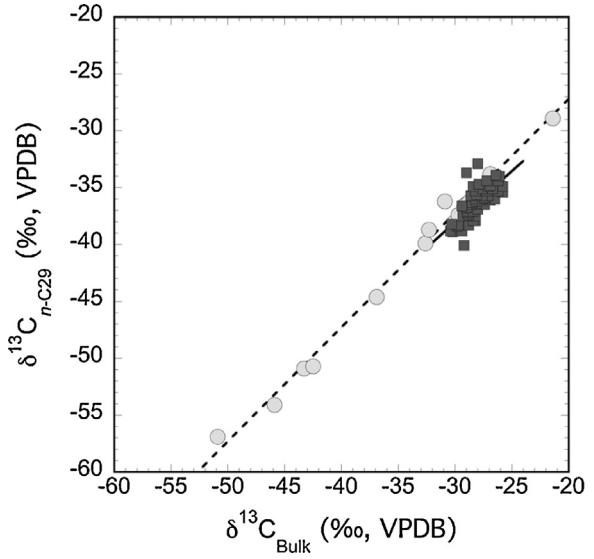

Fig. 2. Cross-plot of the carbon isotope values ($\delta^{13}C$) of n-C_{29} extracted from inflorescences versus bulk inflorescence materials. The hashed and solid lines indicate regressions for plants grown within enclosed and open- environments, respectively. The regressions for plants grown within enclosed and field environments are $\delta^{13}C_{n\text{-}C29} = 1.0 \times \delta^{13}C_{Bulk} - 7.2‰$ and $\delta^{13}C_{n\text{-}C29} = 0.90 \times \delta^{13}C_{Bulk} - 11.1‰$, respectively.

and

$$\text{Open}: \delta^{13}C_{n-C29} = 0.90 \times \delta^{13}C_{Bulk} - 11.1‰ (r^2 = 0.50,\ F_{1,56} = 55.1,\ p < 0.0001), \quad (7)$$

respectively.

When compared, we found the slopes for $\delta^{13}C_{n\text{-}C29}$ values versus $\delta^{13}C_{Bulk}$ for the two growth settings were not different ($p > 0.01$) from one another (Fig. 2). However, the intercepts for these regressions were different and thus, cannot be described by a single slope and intercept, possibly suggesting different behaviors between the two growth settings.

We found the apparent fractionation (ε_{app}) between n-C_{29} extracted from *Cannabis sp*. inflorescence and bulk *Cannabis sp*. inflorescence materials ranged between −11.3‰ and −4.8‰ in individual samples, with an average of −8.0 ± 1.1‰ (n = 72, Table 1). A significant difference was found between the plants grown within an enclosed system versus those grown in field environments [Welch t-test, $t(27.0) = 3.869$, $p = 0.0006$], where the plants grown in enclosed environments had a more positive ε_{app} value (−7.3 ± 0.8‰, n = 15) compared to the plants grown in open-field environments (−8.2 ± 1.0‰, n = 57) (Table 2).

Considering the distinction in absolute abundance of n-alkanes between the plants grown under the two growth settings, an apparent difference in fractionation may not be unexpected. Bulk isotope analysis represents a cumulative measurement of all chemical constituents and tissues, whereas compound-specific isotope analysis explicitly isolates individual components from the bulk material. The compounds of interest to this study are the n-alkyl lipids, but *Cannabis sp*. is well known for producing a wide variety of other compounds, particularly terpenoids [27,30,32]. The carbon isotopic fractionation during terpenoid biosynthesis has been characterized and the apparent fractionation during biosynthesis, as measured relative to bulk leaf tissue, is significantly less negative than that observed for n-alkyl lipids [24,64]. Considering the bulk isotope measurement represents the analysis of a complex mixture of these compounds, in addition to many others with less understood carbon isotopic signatures and fractionations, it should be expected that the bulk $\delta^{13}C$ value of *Cannabis sp*. might be significantly influenced by variations in the combination of these chemical components. As we do not have direct measurements of source CO_2, we cannot assess if the difference in ε_{app} between n-alkanes extracted from *Cannabis sp*. inflorescence and bulk *Cannabis sp*. inflorescence materials for the two growth environments is affected by the environmental conditions or if the difference is due to variation in chemical composition. Nonetheless, these data support the notion that compound-specific isotope measurements provide a more direct measure of plant biochemistry and ecology than bulk isotope measurements.

3.4. *Potential applications of inflorescence* n-*alkanes in emerging marijuana markets*

Stable isotope analyses of marijuana have a demonstrated potential to improve the forensic and law enforcement communities' understanding of marijuana production methods, growth setting, and trafficking networks [1–3]. In this respect, $\delta^{13}C$—as well as nitrogen ($\delta^{15}N$)—isotope values have proven moderately useful. In a series of papers, Shibuya and colleagues demonstrated the potential to differentiate three of the five major production regions of marijuana cultivation in Brazil based on observed differences in the bulk $\delta^{13}C$ and $\delta^{15}N$ isotope values of seized marijuana samples [4,5]. West and others followed with a study of eradicated and seized material from the U.S., but could not to distinguish region-of-origin based on bulk $\delta^{13}C$ and $\delta^{15}N$ values alone [6]. While growth location could not be assigned in that work, the growth setting could be identified using $\delta^{13}C$ values of bulk materials (e.g., leaves, inflorescence) as plants grown in field environments had unique values compared to plants grown within an enclosed glass/hothouse system [6]. Following on these findings, Hurley et al. [38] developed a cultivation model that defined theoretical cut-off limits for $\delta^{13}C_{Bulk}$, where values more negative than −32.0‰ were indicative of *Cannabis* plants grown in enclosed environments and values more positive than −29.0‰ were plants grown outdoors. Values more positive than −32.0‰ and more negative than −29.0‰ could either be assigned to plants grown outdoors in shaded conditions or indoors [38]. The authors found in a blind test of their model that 88% of indoor-grown plants were correctly identified as to growth setting, while 98% of outdoor-grown plants were correctly assigned [38].

We tested the application of this cultivation model to $\delta^{13}C_{n\text{-}C29}$ values measured in the current study by converting $\delta^{13}C_{n\text{-}C29}$ values to $\delta^{13}C_{Bulk}$ values using ε_{app} (Fig. 3). Assignments of plants cultivated in enclosed environments, shaded or enclosed environments, or open-field environments were made for all 72 specimens using the average ε_{app} values between n-C_{29} and *Cannabis* inflorescences from this study. Here, when the average ε_{app} value between *Cannabis* n-C_{29} and inflorescence is used (−8.0‰), we found that 7 plants were assigned to enclosed environments, 20 were assigned to either shaded open environments or enclosed environments, and 45 were assigned to open-field environments. In this exercise, 98% of the *Cannabis* plants cultivated outdoors were correctly identified and 60% of the plants cultivated in enclosed environments were assigned correctly. These finding indicate that by using the *Cannabis* inflorescence-specific ε_{app} value for calculating $\delta^{13}C_{Bulk}$ values and assigning growth setting, the overall reliability of the model was 90%.

The $\delta^{13}C$ values presented here for the n-C_{29} from inflorescence suggest that the cultivation environment (e.g., growth setting) could be established with either bulk plant material or compound-specific inflorescence $\delta^{13}C$ values. However, increased reliability may be achieved if inflorescence n-alkane concentrations and distributions were included in the model allowing for a growth setting-specific ε_{app} value to be used (Table 2). A specific advantage

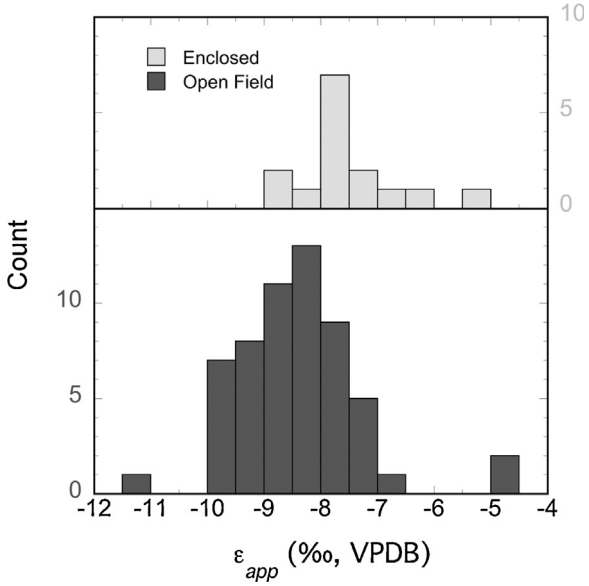

Fig. 3. Histogram of the apparent fractionation (ε_{app}) between n-C_{29} extracted from inflorescence versus bulk inflorescence measured for the same sample. Specimens grown within enclosed environments are shown in light gray, while plants grown in field environments are shown in dark gray.

of compound-specific isotope measurements is that bulk tissue is not needed to make an isotopic measurement and these specific compounds may be isolated from complex mixtures such as *Cannabis* extracts and infused products. While stringently controlled growth experiments are needed to determine the specific mechanism(s) behind the findings presented here, this study of plants grown in "real world" settings demonstrates that the carbon isotope ratios and concentrations of *n*-alkanes have the potential to become an ideal regulatory tool to establish growth settings of *Cannabis* materials post-harvest.

4. Conclusions

Here we presented chain-length distributions and concentrations of *n*-alkanes, plus carbon isotope ratios of *n*-C_{29} extracted from *Cannabis sp.* inflorescences. We found chain-length distributions, concentrations, and carbon isotope variations of these lipids relate to growth setting. While the biosynthetic mechanism is unknown, we found that inflorescences of *Cannabis sp.* have nearly 3-times the concentrations of *n*-alkanes than the average angiosperm leaf and that *Cannabis* plants grown under field conditions have significantly more inflorescence *n*-alkanes relative to plants grown within enclosed environments. Carbon isotope ratios of *Cannabis sp.* inflorescences largely reflect the isotope composition of source CO_2, which can vary greatly in enclosed environments. In addition, we found that *n*-alkanes from *Cannabis* plants grown within enclosed environments had a large range of $\delta^{13}C$ values with both the most positive and most negative $\delta^{13}C$ values in this dataset reported.

Together, these findings suggest that inflorescence lipid distributions, concentrations, and carbon isotope values have the potential to be used to identify the growth setting of *Cannabis sp.* plants. As legal and illegal commercial production of marijuana increase, the need to establish the growth setting of *Cannabis* will also increase. Many communities, municipalities, and jurisdictions in the U.S. where *Cannabis* production is legal now regulate how the product is grown. With additional jurisdictions seeking to legalize marijuana usage for both medical and recreational purposes, state and federal regulators will need additional analytical tools to certify a *Cannabis* product's cultivation setting. In this study, we show that distributions, concentrations, and carbon isotopic variations in specific inflorescence lipids are related to growth setting and suggest that this tool may potentially be useful to support or refute a producer's claim regarding the growth setting of *Cannabis*.

Acknowledgements

Mahmoud ElSohly and Zlatko Mehmedic at the University of Mississippi provided *Cannabis* specimens and collection information. B. J. T. and J. R. E. designed experiment. B. H. and J. E. B. performed extractions and purifications and provided intellectual input and contributed to the text. B. J. T. and B. H. carried out concentration and compound-specific isotope analysis. L. A. C. and J. R. E. provided intellectual input, support, and contributed to the text. B. J. T. was the lead in writing this work. This contribution was strengthened by comments from two anonymous reviewers. This research was supported by NSF-ISO-1052551 awarded to J. R. E. and B. J. T.

References

[1] J.H. Liu, W.F. Lin, M.P. Fitzgerald, S.C. Saxena, Y.N. Shieh, Possible characterization of samples of *Cannabis sativa* L. by their carbon isotopic distributions, J. Forensic Sci. 24 (1979) 814–816.
[2] T.M. Denton, S. Schmidt, C. Critchley, G.R. Stewart, Natural abundance of stable carbon and nitrogen isotopes in *Cannabis sativa* reflect growth conditions, Aust. J. Plant Physiol. 28 (2001) 1005–1012.
[3] E.M. Galimov, V.S. Sevastyanov, E.V. Kulbachevskaya, A.A. Golyavin, Isotope ratio mass spectrometry: $\delta^{13}C$ and $\delta^{15}N$ analysis for tracing the origin of illicit drugs, Rapid Commun. Mass Spectrom. 19 (2005) 1213–1216.
[4] E.K. Shibuya, J.E.S. Sarkis, O. Negrini-Neto, L.A. Martinelli, Carbon and nitrogen stable isotopes as indicative of geographical origin of marijuana samples seized in the city of San Paulo, Brazil, Forensic Sci. Int. 167 (2007) 8–15.
[5] E.K. Shibuya, J.E.S. Sarkis, O. Negrini-Neto, M.Z. Moreira, R.L. Victoria, Sourcing Brazilian marijuana by applying IRMS analysis to seized samples, Forensic Sci. Int. 160 (2006) 35–43.
[6] J.B. West, J.M. Hurley, J.R. Ehleringer, Stable isotope ratios of marijuana. I. Carbon and nitrogen stable isotopes describe growth conditions, J. Forensic Sci. 54 (2009) 84–89.
[7] G.D. Farquhar, J.R. Ehleringer, K.T. Hubick, Carbon isotope discrimination and photosynthesis, Annu. Rev. Plant Physiol. Mol. Biol. 40 (1989) 503–537.
[8] M.N. O'Leary, Carbon isotope fractionation in plants, Phytochemistry 20 (1981) 553–567.
[9] D.J. Beerling, J. Heath, F.I. Woodward, T.A. Mansfield, Drought-CO_2 interactions in trees: observations and mechanisms, New Phytol. 134 (1996) 235–242.
[10] J.R. Ehleringer, Correlations between carbon isotope discrimination and leaf conductance to water vapor in common beans, Plant Physiol. 93 (1990) 1422–1425.
[11] J.R. Ehleringer, T.E. Cerling, Atmospheric CO_2 and the ratio of intercellular to ambient CO_2 concentrations in plants, Tree Physiol. 15 (1995) 105–111.
[12] G.D. Farquhar, M.C. Ball, S. von Caemmerer, Z. Roksandic, Effect of salinity and humidity on $\delta^{13}C$ value of halophytes- evidence for diffusional isotope fractionation determined by the ratio of intercellular-atmospheric partial pressure of CO_2 under different environmental conditions, Oecologia 52 (1982) 121–124.
[13] G.D. Farquhar, E.D. Schulze, M. Kuppers, Responses to humidity by stomata of *Nicotiana glauca* L. and *Corylus avellana* L. are consistent with the optimization of carbon dioxide uptake with respect to water loss, Aust. J. Plant Physiol. 7 (1980) 315–327.
[14] G.D. Farquhar, T.A. Sharkey, Stomatal conductance and photosynthesis, Annu. Rev. Plant Physiol. Mol. Biol. 33 (1982) 317–345.
[15] J.A. Panek, R.H. Waring, Carbon isotope variation in Douglas-fir foliage: improving the $\delta^{13}C$-climate relationship, Tree Physiol. 15 (1995) 657–663.
[16] J.P. Sparks, J.R. Ehleringer, Leaf carbon isotope discrimination and nitrogen content for riparian trees along elevational transects, Oecologia 109 (1997) 362–367.
[17] K. Winter, J.A.M. Holtum, G.E. Edwards, M.H. O'Leary, Effect of low relative humidity on $\delta^{13}C$ value in two C_3 grasses and in Panicum milioides, a C_3-C_4 intermediate species, J. Exp. Bot. 33 (1982) 88–91.
[18] J. Zimmerman, J.R. Ehleringer, Carbon isotope ratios are correlated with irradiance levels in the Panamanian orchid *Catasetum viridiflavum*, Oecologia 83 (1990) 247–249.
[19] J.R. Ehleringer, C.B. Field, Z.F. Lin, C.Y. Kuo, Leaf carbon isotope and mineral composition in subtropical plants along an irradiance cline, Oecologia 70 (1986) 520–526.
[20] B.N. Smith, J. Oliver, C. McMillan, Influence of carbon source, oxygen concentration, light intensity, and temperature on C^{13}/C^{12} ratios in plant tissues, Bot. Gazette 137 (1976) 99–104.
[21] H. Yang, M. Pagani, D.E.G. Briggs, M.A. Equiza, R. Jagels, Q. Leng, et al., Carbon and hydrogen isotope fractionation under continuous light: implications for paleoenvironmental interpretations of the High Arctic during Paleogene warming, Oecologia 160 (2009) 461–470.
[22] R.W. Pearcy, W.A. Pfitsch, Influence of sunflecks on the ^{13}C of *Adenocaulon bicolor* plants occurring in contrasting forest understory microsites, Oecologia 86 (1991) 457–462.
[23] N. Pedentchouk, W. Sumner, B.J. Tipple, M. Pagani, $\delta^{13}C$ and δD compositions of n-alkanes from modern angiosperms and conifers: an experimental set up in central Washington State, USA, Org. Geochem. 39 (2008) 1066–1071.
[24] A.F. Diefendorf, K.H. Freeman, S.L. Wing, H.V. Graham, Production of *n*-alkyl lipids in living plants and implications for the geologic past, Geochim. Cosmochim. Acta 75 (2011) 7472–7485.
[25] A.F. Diefendorf, K.E. Mueller, S.L. Wing, P.L. Koch, K.H. Freeman, Global patterns in leaf ^{13}C discrimination and implications for studies of past and future climate, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 5738–5743.
[26] P. Tipparat, W. Kunkaew, S. Julsrigival, S. Pinmanee, S. Natakankitkul, Classification of cannabis plants grown in Northern Thailand using physico-chemical properties, J. Nat. Sci. Res. 4 (2014) 46–54.
[27] M.A. ElSohly, S.A. Ross, Z. Mehmedic, R. Arafat, B. Yi, Banahan III BF. Potency trends of Δ^9-THC and other cannabinoids in confiscated marijuana from 1980-1997, J. Forensic Sci. 45 (2000) 24–30.
[28] S. Chandra, H. Lata, Z. Mehmedic, I.A. Khan, M.A. ElSohly, Assessment of Cannabinoids content in micropropagated plants of *Cannabis sativa* and their comparison with conventionally propagated plants and mother plants during developmental stages of growth, Planta Med. 76 (2010) 743–750.
[29] Y. Ilias, S. Rudaz, P. Mathieu, P. Christen, J.L. Veuthey, Extraction and analysis of different cannabis samples by headspace solid-phase microextraction combined with gas chromatography–mass spectrometry, J. Sep. Sci. 28 (2005) 2293–2300.
[30] J.T. Fischedick, A. Hazekamp, T. Erkelens, Y.H. Choi, R. Verpoorte, Metabolic fingerprinting of *Cannabis sativa* L., cannabinoids and terpenoids for chemotaxonomic and drug standardization purposes, Phytochemistry 71 (2010) 2058–2073.
[31] Z. Muccio, C. Wockel, Y. An, G.P. Jackson, Comparison of bulk and compound-specific $\delta^{13}C$ isotope ratio analysis for the discrimination between Cannabis samples, J. Forensic Sci. 57 (2011) 757–764.

[32] R.P. Latta, B.J. Eaton, Seasonal fluctuations in cannabinoid content of Kansas Marijuana, Econ. Bot. 29 (1975) 153–163.
[33] I. Barni-Comparini, S. Ferri, F. Centini, Cannabinoid level in the leaves as a tool for the early discrimination of cannabis chemovariants, Forensic Sci. Int. 24 (1984) 37–42.
[34] K. Mechtler, J. Bailer, K. de Hueber, Variations in the Δ^9-THC content in single plant of hemp varieties, Ind. Crops Prod. 19 (2004) 19–24.
[35] J.R. Burgdorf, B. Kilmer, R.L. Pacula, Heterogeneity in the composition of marijuana seized in California, Drug Alcohol Depend. 117 (2011) 59–61.
[36] G. Eglinton, R.J. Hamilton, Leaf epicuticular waxes, Science 156 (1967) 1322–1335.
[37] A. Schimmelmann, A.L. Sessions, M. Mastalerz, Hydrogen isotopic (D/H) composition of organic matter during diagenesis and thermal maturation, Annu. Rev. Earth Planet. Sci. 34 (2006) 501–533.
[38] J.M. Hurley, J.B. West, J.R. Ehleringer, Stable isotope models to predict geographic origin and cultivation conditions of marijuana, Sci. Justice 50 (2010) 86–93.
[39] J.B. West, J.M. Hurley, F.O. Dudas, J.R. Ehleringer, The stable isotope ratios of marijuana. II. Strontium isotopes relate to geographic origin, J. Forensic Sci. 54 (2009) 1261–1269.
[40] J.M. Hurley, J.B. West, J.R. Ehleringer, Tracing retail cannabis in the United States: geographic origin and cultivation patterns, Int. J. Drug Policy 21 (2010) 222–228.
[41] B.J. Tipple, M. Pagani, Environmental control on eastern broadleaf forest species' leaf wax distributions and D/H ratios, Geochim. Cosmochim. Acta 111 (2013) 64–77.
[42] R. Marzi, B.E. Torkelson, R.K. Olson, A revised carbon preference index, Org. Geochem. 20 (1993) 1303–1306.
[43] R.T. Bush, F.A. McInerney, Leaf wax *n*-alkane distributions in and across modern plants: Implications for paleoecology and chemotaxonomy, Geochim. Cosmochim. Acta 117 (2013) 161–179.
[44] B. Shu, L. Lin, Y. Zhang, H. Wang, H. Luo, N-alkane profiles of common rangeland species in northern China and the influence of drying method on their concentrations, Can. J. Plant Sci. 88 (2008) 137–141.
[45] B. Gamarra, A. Kahmen, Concentrations and δ^2H values of cuticular n-alkanes vary significantly among plant organs, species and habitats in grasses from an alpine and a temperate European grassland, Oecologia 178 (2015).
[46] F. Rommerskirchen, A. Plader, G. Eglinton, Y. Chikaraishi, J. Rullkotter, Chemotaxonomic significance of distribution and stable carbon isotopic composition of long-chain alkanes and alkan-1-ols in C_4 grass waxes, Org. Geochem. 37 (2006) 1303–1332.
[47] M. Riederer, C. Markstadter, Cuticular waxes: a critical assessment of current knowledge, in: G. Kersteins (Ed.), Plant Cuticles: An Integrated Functional Approach, BIOS Scientific Publishers, Oxford, 1996, pp. 189–200.
[48] P.G. Guiz, Epicuticular leaf waxes in the evolution of the plant kingdom, J. Plant Physiol. 143 (1994) 453–464.
[49] M.J. Lockheart, P.F. Van Bergen, R.P. Evershed, Variation in the stable carbon isotope composition of individual lipids from the leaves of modern angiosperms: implications for the study of higher land plant-derived sedimentary organic matter, Org. Geochem. 26 (1997) 137–153.
[50] E. Piasentier, S. Bovolenta, F. Malossini, The *n*-alkane concentrations in buds and leaves of browsed broadleaf trees, J. Agric. Sci. 135 (2000) 311–320.
[51] T. Shepherd, D.W. Griffiths, The effects of stress on plant cuticular waxes, New Phytol. 171 (2006) 469–499.
[52] D. Sachse, J. Radke, G. Gleixner, δD values of individual *n*-alkanes from terrestrial plants along a climatic gradient – implications for the sedimentary biomarker record, Org. Geochem. 37 (2006) 469–483.
[53] M. Riederer, G. Schneider, The effect of the environment on the permeability and composition of *Citrus* leaf cuticle, Planta 180 (1990) 154–165.
[54] K.A. Hughen, T.I. Eglinton, L. Xu, M. Makou, Abrupt tropical vegetation response to rapid climate changes, Science 304 (2004) 1955–1959.
[55] E. Schefuss, V. Ratmeyer, J.B.W. Stuut, J.H.F. Jansen, J.S. Sinninghe Damste, Carbon isotope analyses of *n*-alkanes in dust from the lower atmosphere over the central eastern Atlantic, Geochim. Cosmochim. Acta 67 (2003) 1757–1767.
[56] F. Rommerskirchen, G. Eglinton, L. Dupont, U. Guntner, C. Wenzel, J. Rullkotter, A north to south transect of Holocene southeast Atlantic continental margin sediments: relationship between aerosol transport and compound-specific δ^{13}C land plant biomarker and pollen records, Geochem. Geophys. Geosyst. 4 (2003) 1101.
[57] A. Vogts, H. Moossen, F. Rommerskirchen, J. Rullkotter, Distribution patterns and stable carbon isotopic composition of alkanes and alkan-1-ols from plant waxes of African rain forest and savanna C_3 species, Org. Geochem. 40 (2009) 1037–1054.
[58] A. Vogts, E. Schefuss, T. Badewien, J. Rullkotter, *n*-Alkane parameters from a deep sea sediment transect off southwest Africa reflect continental vegetation and climate conditions, Org. Geochem. 47 (2012) 109–119.
[59] T. Shepherd, G.W. Robertson, D.W. Griffiths, A.N.E. Birch, G. Duncan, Effects of environment on the composition of epicuticular wax from kale and swede, Phytochemistry 40 (1995) 407–417.
[60] B.J. Tipple, M. Pagani, A 35 Myr North American leaf-wax compound-specific carbon and hydrogen isotope record: implications for C_4 grasslands and hydrologic cycle dynamics, Earth Planet. Sci. Lett. 299 (2010) 250–262.
[61] D.E. Pataki, S.E. Bush, J.R. Ehleringer, Stable isotopes as a tool in urban ecology, in: L.B. Flanagan, J.R. Ehleringer, D.E. Pataki (Eds.), Stable Isotopes and Biosphere – Atmosphere Interactions: Processes and Biological Controls, Academic Press, 2005, p. 400.
[62] D.E. Pataki, T. Xu, Y.Q. Luo, J.R. Ehleringer, Inferring biogenic and anthropogenic CO_2 sources across an urban to rural gradient, Oecologia 152 (2007) 307–322.
[63] P. Deines, The isotopic composition of reduced organic carbon, in: P. Fritz, J.C. Fontes (Eds.), Handbook of Environmental Isotope Geochemistry: I The Terrestrial Environment, Elsevier, Amsterdam, 1980, pp. 329–406.
[64] A.F. Diefendorf, K.H. Freeman, S.L. Wing, Distribution and carbon isotope patterns of diterpenoids and triterpenoids in modern temperate C_3 trees and their geochemical significance, Geochim. Cosmochim. Acta 85 (2012) 342–356.
[65] B.R. Bondada, B.M. Oosterhuis, J.B. Murphy, K.S. Kim, Effect of water stress on the epicuticular wax composition and ultrastructure of cotton (Gossypium hirsutum L.) leaf, bract and boll, Environmental and Experimental Botany 36 (1996) 61–69.
[66] R. Gonzalez, N.D. Paul, K. Percy, M. Ambrose, C.K. McLaughlin, J.D. Barnes, M. Areses, A.R. Wellburn, Responses to ultraviolet-B radiation (280-315 nm) of pea (Pisum sativum) lines differing in leaf surface wax, Physiologia Plantarum 98 (1996) 852–860.
[67] J.D. Barnes, K.E. Percy, N.D. Paul, P. Jones, C.K. McLaughin, P.M. Mullineaux, G. Creissen, A.R. Wellburn, The influence of UV-B radiation on the physiochemical nature of tobacco (Nicotiana tabacum L.) leaf surfaces, J. Exp. Bot. 47 (1996) 99–109.
[68] E.A. Baker, The Influence of environment on leaf wax development in Brassica oleracea var. gemmifera, New Phytologist 73 (1974) 955–966.
[69] K.E. Percy, C.S. Awmack, R.L. Lindroth, M.E. Kubiske, B.J. Kopper, J.G. Isebrands, K.S. Pregitzer, G.R. Hendrey, R.E. Dickson, D.R. Zak, E. Oksanen, J. Sober, R. Harrington, D.F. Karnosky, Altered performance of forest pests under atmospheres enriched by CO_2 and O_3, Nature 420 (2002) 403–407.
[70] M. Dixon, C. LeThiec, J.P. Garrec, An investigation into the effects of ozone and drought, applied singly and in combination, on the quantity and quality of the epicuticular wax of Norway spruce, Plant Physiology and Biochemistry 35 (1997) 447–454.
[71] A. Sauer, K.-P. Heise, On the light dependence of fatty acid synthesis in Spinach chloroplasts, Plant Physiol. 73 (1983) 11–15.
[72] Y. Sasaki, A. Kozaki, M. Hatano, Link between light and fatty acid synthesis: Thioredoxin-linked reductive activation of plastidic acetyl-CoA carboxylase, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 11096–11101.
[73] T. Shepherd, G.W. Robertson, D.W. Griffiths, A.N.E. Birch, Effects of environment on the composition of epicuticular wax esters from kale and swede, Phytochemistry 46 (1997) 83–96.